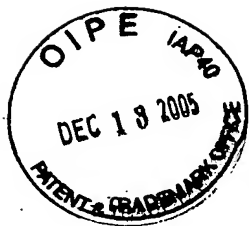


09/354,109



Patent
Attorney's Docket No. 010091-056

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

REQUEST FOR FILING CONTINUATION/DIVISIONAL
APPLICATION UNDER 37 C.F.R. § 1.53(b)

Box PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a request for filing a ☐ continuation ☒ divisional application under 37 C.F.R. § 1.53(b) of pending Application No. 08/957,327 filed on October 24, 1997, for LIPOSOMES CONTAINING OLIGONUCLEOTIDE, by the following named inventor(s):

- (a) Full Name Usha Kasid
- (b) Full Name Prafulla Gokhale
- (c) Full Name Anatoly Dritschilo
- (d) Full Name Aquilur Rahman

☒ The entire disclosure of the prior application from which a copy of the oath or declaration is supplied herewith is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

☐ This application is being filed by less than all the inventors named in the prior application. In accordance with 37 C.F.R. 1.63(d)(2), the Commissioner is requested to delete the name(s) of the following person or persons who are not inventors of the invention being claimed in this application.

- (a) Full Name _____
- (b) Full Name _____
- (c) Full Name _____

1. ☒ Enclosed is a copy of the prior Application No. 08/957,327 as originally filed on October 24, 1997, including copies of the specification, claims, drawings and the executed oath or declaration as filed.
2. ☐ Enclosed is a revised prior application and a copy of the prior executed oath or declaration as filed. No new matter has been added to the revised application.
3. ☒ One statement(s) claiming small entity status ☐ are enclosed ☒ were filed in prior Application No. 08/957,327, filed on October 24, 1997.

Handwritten signature and date 11/18/99

(12/97)

4. ☒ The filing fee is calculated below ☒ and in accordance with the enclosed preliminary amendment:

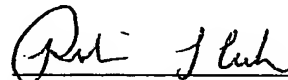
C L A I M S					
	NO. OF CLAIMS		EXTRA CLAIMS	RATE	FEE
Basic Application Fee					\$760.00
Total Claims	16	MINUS 20 =	0	x \$18.00 =	0.00
Independent Claims	1	MINUS 3 =	0	x \$78.00 =	0.00
If multiple dependent claims are presented, add \$260.00					0.00
Total Application Fee					760.00
If small entity status is claimed, subtract 50% of Total Application Fee					380.00
Add Assignment Recording Fee of \$40.00 if Assignment document is enclosed					0.00
TOTAL APPLICATION FEE DUE					380.00

5. ☐ Charge \$ _____ to Deposit Account No. 02-4800 for the fee due.
6. ☒ A check in the amount of \$ 380.00 is enclosed for the fee due.
7. ☒ The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800. This paper is submitted in triplicate.
8. ☐ Cancel in this application original claims __ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)
9. ☒ Amend the specification by inserting before the first line the sentence: --This application is a ☐ continuation, ☒ divisional, of Application No. 08/957,327, filed October 24, 1997, which takes priority from Provisional Application No. 60/041,192, filed March 21, 1997.--
10. ☐ Transfer the drawings from the pending prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate of this paper is enclosed for filing in the prior application file. (May only be used if signed by person authorized under 37 C.F.R. § 1.138 and before payment of issue fee.)

11. ☐ New drawings are enclosed.
12. ☒ Priority of Provisional Application No. 60/041,192 filed on March 21, 1997 in U.S. (country) is claimed under 35 U.S.C. § 119.
13. ☒ A preliminary amendment is enclosed.
14. ☒ Assignee of record is GEORGETOWN UNIVERSITY SCHOOL OF MEDICINE.
15. ☒ Also enclosed Statement Pursuant to 37 C.F.R. §1.822 with a copy of the Sequence Listing; Information Disclosure Statement with PTO-1449.
16. ☒ The power of attorney in the prior application is to ____.
- a. ☒ The power appears in the original papers in the prior application.
 - b. ☒ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.
 - c. ☒ Recognize as Associate Attorney Robin L. Teskin, Reg. No. 35,030.
 - d. ☒ Address all future communications to: (May only be completed by applicant, or attorney or agent of record.)

Robin L. Teskin
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July 15, 1999
Date

By: 
Robin L. Teskin
Registration No. 35,030

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SIGNATOR:

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- ☐ inventor(s)
☐ assignee of complete interest
☒ attorney or agent of record
☐ filed under 37 C.F.R. § 1.34(a)

APPLICATION FOR UNITED STATES PATENT

This application takes priority from Provisional Application 60/041,192 filed March 21, 1997. This work was supported by grants from the National Institutes of Health. The United States Government has certain rights in this invention.

Field of the Invention:

This invention is related to use antisense of sequences of ≤ 40 bases for enhancing radiosensitivity of radiation-resistant tumors and to cationic liposomes which are particularly useful as carriers for antisense sequences.

Background of the invention:

Radiation therapy is an important treatment modality of cancer. However, therapeutic management may be limited by the inherent relative resistance of some cancers to the cytotoxic effects of ionizing radiation. Recently, several lines of investigation have coalesced to demonstrate a link between certain oncogenes (*ras*, *raf*, *cot*, *mos*, *myc*), growth factors (PDGF, FGF) and the phenomenon of cellular resistance to ionizing radiation.

It was previously reported that expression of antisense *c-raf-1* cDNA results in reduced expression (RNA) of *c-raf-1* gene, a cause of delayed tumor growth in athymic mice and in enhanced radiation sensitivity of relatively radioresistant laryngeal squamous carcinoma cells, SQ-20B (Kasid et al., Science 243:1354-1356, 1989).

Summary of the Invention:

It is possible to radiosensitize tumor cells by administration of compositions containing the Human antisense *c-raf-1* oligodeoxyribonucleotide (ODN/oligo) sequence:

5'-GTGCTCCATTGATGC-3' (seq. #1) wherein only the end bases are phosphorylated is a preferred sequence. Antisense sequences of up to 40 bases which containing this sequence may be used in accord with the teachings of this disclosure. A composition of the 25-mer oligo:

5'-CCTGTATGTGCTCCATTGATGCAGC-3' (seq. #2) wherein the sequence is also effective. Compositions comprising cationic liposomes containing at least one non-toxic cationic lipid, phosphatidylcholine and cholesterol may be used as a carrier system.

Description of the Invention:

The search for clinically useful radiation sensitizers for treatment of cancers which fail to respond to radiation therapy has been actively pursued. This invention provides specific sequences which, while inducing radiation sensitivity on tumor cells, is non-toxic to normal tissue. As little as 10 $\mu\text{mol}/\mu\text{l}$ of the sequences encapsulated in liposomes is effective when tumor cells are contacted with the compositions. It was found that the expression and enzymatic activity of Raf-1 protein are inhibited in cells exposed to *raf* antisense oligodeoxyribonucleotide (As-ODNs) directed against the translation initiation site of human *c-raf-1* cDNA. In contrast, treatment of cells with an equimolar concentration of *raf* sense oligodeoxyribonucleotide (S-

ODNs) had no effect on the expression and activity of Raf-1. Furthermore, it was observed radiosensitization of raf As-ODNs-treated SQ-20B cells. The dose modifying factor of As-ODNs treatment was 1.4. This demonstrates that raf As-ODNs is a DNA sequence-specific radiosensitizer which may have potential for use in the radiation therapy of cancers. Hence, the method of the invention comprises administration of a radiosensitizing effective amount of at least one antisense nucleotide of no more than 40 bases containing the sequence 5'-GTGCTCCATTGATGC-3'.

This invention provides new liposomal compositions which provide means of enhancing the effect of oligonucleotides encapsulated in the novel liposomes. The invention is exemplified using encapsulated raf oligodeoxyribonucleotides. The novel cationic liposomes of the invention were prepared using dimethyldioctadecyl ammonium bromide, phosphatidylcholine and cholesterol. However, other nontoxic cationic lipids such as N-(2,3-(dioleoyloxy)propyl)-N,N,N-trimethyl ammonium chloride or 1-[2-(9(Z)-octadecenoyloxy)-ethyl]-2-(8(Z)heptadecenyl)-3-(2-hydroxyethyl)-imidazolinium chloride may be used. These liposomes provide protection from degradation in plasma and normal tissues to protect the oligonucleotides while they are reaching their intended target cells. Hence, smaller amounts of oligonucleotides are needed to obtain desired results.

Cationic liposomes have been used to deliver genes *in vitro* and *in vivo* (Felgner, Editorial, Human Gene Therapy 7:1791-1793, 1996). The novel formulation of the cationic liposomes to

encapsulate antisense *raf* oligonucleotides has been tested and found effective. It has been found that these liposomes encapsulate >90% oligos. Liposomal encapsulation provides protection of antisense *raf* oligonucleotide from degradation in plasma, and normal tissues, and that tumor cells treated with the liposome-encapsulated antisense *raf* oligo (LE-ATG-AS *raf* ODN) are significantly radiosensitive compared to control or sense *raf* oligo-treated cells. It is now disclosed herein that LE-ATG-AS *raf* ODN inhibits Raf-1 protein expression in solid tumors. (Gokhale et al., "Antisense 97: Targeting the Molecular Basis of Disease," Cambridge Symposium Meeting, May 1997). The liposomal compositions of the invention disclosed herein are believed to be particularly useful as radiosensitizers in solid tumors.

Materials and methods

Oligodeoxyribonucleotides

The sense and antisense *raf* ODNs were designed against the translation initiation site of human *c-raf-1* cDNA in accord with the teachings of Bonner (Bonner et al., Nucleic Acids Res., 14:1009-1015, 1986), and have the following sequence: sense ODN (ATG-S *raf*), 5'-GCATCAATGGAGCAC-3' (seq. #3); antisense ODN (ATG-AS *raf*), 5'-GTGCTCCATTGATGC-3' (seq. #1), Only two of the bases, one at each end, are phosphorothioated. While antisense sequences of *raf* of up to 40 bases containing seq. #1 may be used, the larger sequences may be less effective. The fully phosphorothioated sequences may also be effective, but are more

likely to cause toxic effects. That the sequences having only the end bases phosphorothioated are non-toxic to normal cells greatly enhances the value of such sequences for use in targeting malignant cells.

5 Synthesis and purification of ODNs

Oligodeoxyribonucleotide synthesis was performed at Lofstrand Labs Limited, Gaithersburg, using Beta-Cyanoethyl Phosphoramidite chemistry on Biosearch 8750 DNA synthesizers. Desired base linkages were modified to phosphorothioate groups using 3H-1,2-benzodithiole-3-1,1,1-dioxide as the sulfurizing agent. Oligos synthesized at the 15 μ mol scale were cleaved and deprotected in 30% ammonium hydroxide for 24 hours at room temperature and purified over reverse phase chromatography columns. Deprotected DMT-On (trytl on) oligos retained by the support column as failure sequences were washed off in basic aqueous solution. Full length product was detritylated using 2% trifluoro-acetic acid, washed with sterile, deionized water and eluted with 20% acetonitrile, dried and resuspended in sterile, deionized water. For quality control, a small aliquot of each oligo preparation was ³²P-end labeled and visualized by polyacrylamide gel electrophoresis (20% acrylamide and 5% bis) followed by densitometer scanning of the labeled products.

Source of Cells

The SQ-20B tumor cells used were established in culture from the laryngeal squamous carcinoma of a patient who had failed a full course of radiation therapy. *In vitro* radiation survival

analysis has confirmed that these tumor cells are relatively radioresistant. Previously, it had been demonstrated that the transfection of antisense human *c-raf-1* cDNA into SQ-20B cells leads to the down-regulation of endogenous *raf-1* gene expression, delayed tumor growth in athymic mice, and enhanced radiation sensitivity compared with the sense *c-raf-1* cDNA transfectants, and the untransfected tumor cells. Hence a "dual" role was proposed for Raf-1 in these tumor cells; a direct role in the expression of the malignant phenotype, and an indirect role in cellular responses to radiation damage.

Cell culture, cell viability and cell cycle assay

SQ-20B stock cultures were grown and maintained in complete Dulbecco's modified MEM (GIBCO/BRL) containing 20% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM non-essential amino acids, 0.4 µg/ml hydrocortisone, 100 µg/ml streptomycin and, 100 U/ml penicillin. For cell viability and cell cycle analysis, logarithmically growing cells were cultured in T-25 flasks, complete medium was replaced with 1% FBS containing medium in the presence of a desired concentration of ODNs followed by continued incubation for various times. Control cells were grown in 1% FBS containing medium without ODNs. Cells were collected by trypsinization and viability was determined by the trypan blue dye exclusion assay. Duplicate samples were analyzed by the FACS method to determine the % distribution of cells in different phases of the cell cycle.

Intracellular uptake and stability of ODNs

Stock solutions of oligos were prepared by reconstitution of the lyophilized compounds in sterile phosphate buffered saline (PBS) just before use. Oligos (10 pmol/ μ l) were 5'-end labeled with [γ - 32 P]ATP (50 μ Ci, 3000 Curies/mmol) and T4 polynucleotide kinase (10 U/ μ l), and purified on ChromaSpin-10 column (Clontech). Logarithmically growing cells were rinsed with Hank's balanced salt solution and fresh medium containing 1% FBS, 100 pmol/ μ l ODNs, and radiolabeled ODNs (2×10^6 cpm) was added. Following incubation at 37°C in a humidified, 5% CO₂ atmosphere for the desired time, cells were collected, washed three times in PBS, and lysed for 2 hours at 37°C in the buffer containing 10 mM Tris-HCl (pH 7.5), 1% SDS, and 200 μ g/ml proteinase K. DNA isolation was performed using the phenol:chloroform:isoamylalcohol (25:24:1) extraction procedure. ODNs uptake was determined by liquid scintillation counting of the aqueous phase, and was expressed as a percentage of the total radioactivity applied to the cells. Aliquots of the cell extracts (5×10^3 cpm per sample) were resuspended in sample buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue) and electrophoresed on 8 M Urea-20% polyacrylamide gel. The dried gel was autoradiographed to visualize the oligos.

Raf-1 immunoprecipitation, immunoblotting, and *in vitro* kinase activity assays

Cells were washed twice with cold PBS and lysed at 4°C for 10 min. in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium

deoxycholate, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20µg/ml aprotinin, 20µg/ml leupeptin). Insoluble material was removed by centrifugation at 4°C for 30 min at 9,000 xg and protein concentration was determined (Pierce). ● Immunoprecipitation was performed by incubating the lysate with polyclonal Raf-1 antibody directed against the last 12 amino acids of human Raf-1 and conjugated with Protein A-Agarose (Santa Cruz). Immunoprecipitates were washed once with the lysis buffer, twice with 0.5M LiCl-0.1M Tris (pH7.4), and once with 10 mM Tris (pH 7.4). For immunoblotting, the immune-complex was boiled in Lammeli sample buffer and electrophoresed on a 7.5% SDS-polyacrylamide gel, followed by western blotting using Raf-1 antibody and detection of Raf-1 protein by the ECL method according to the manufacturer's protocol (Amersham). Raf-1 protein kinase activity was determined *in vitro* using a pseudosubstrate peptide (Syntide 2, Santa Cruz) as substrate. The phosphotransferase assay was performed by incubating Raf-1 immunoprecipitates along with the exogenous substrate for 20 min at 30°C in 40 µl of reaction buffer containing 25 mM HEPES (pH7.4), 25 mM 8 glycerol phosphate, 1 mM DTT, 10 mM MnCl₂, 100 µM ATP, and 10 µCi of [³²P]ATP. The assay was terminated by spotting 20 µl of the reaction mix onto 2 cm x 3 cm pieces of Whatman P81 phosphocellulose paper. The filters were washed four times for 15 min in a solution of 0.85% phosphoric acid. The Syntide 2-associated ³²P radioactivity bound to the filters was quantitated by Cerenkov counting.

Clonogenic radiation survival assay, and data analysis

The appropriate number of tumor cells were seeded into T-25 flasks in complete medium containing 20% FBS. Cells were allowed to attach for 8 hr and the medium was replaced with medium containing 1% FBS and 100 pmol/ μ l of *raf* S- or AS-ODNs. Control cells were incubated with complete medium containing 1% FBS. Oligo treatment lasted for 10 hr, followed by exposure of the cells to the indicated graded doses of γ -radiation. Irradiations were performed using a ^{137}Cs gamma irradiator (JL Shepard MARK-I irradiator) at a dose rate of 3.83 Gy/min. The irradiated cells were then maintained under these incubation conditions for an additional 2 hr. The growth medium in all flasks then was replaced with complete medium containing 20% FBS and the cells were incubated for 7-10 days. Surviving colonies were fixed and stained with 1% methylene blue. Colonies greater than 50 cells were scored and the data were fitted to the Albright's computer-generated single-hit multitarget and linear-quadratic models of radiation survival response.

Liposome preparation

Liposome-encapsulated *raf* oligodeoxyribonucleotides, LE-ATG-S *raf* ODN and LE-ATG-AS *raf* ODN, were prepared using dimethyldioctadecyl ammonium bromide, phosphatidylcholine and cholesterol (Avanti Polar Lipids, Inc., Alabaster, AL, USA) in a molar ratio of 1:3.2:1.6. Briefly, the lipids dissolved in chloroform or methanol were evaporated to dryness in a round-bottomed flask using a rotatory vacuum evaporator. The dried lipid film was

hydrated overnight at 4°C by adding 1 ml of ODN at 1.0 mg/ml in phosphate buffered saline (PBS). The film was dispersed by vigorous vortexing and the liposome suspension was sonicated for 5 min in a bath type sonicator (Laboratory Supplies Co. Inc., Hicksville, NY, USA). The ODN to lipid ratio was 30 µg ODN/mg of lipid. The unencapsulated ODN was removed by washing the liposomes by centrifugation (3 times at 75,000 g for 30 min) in PBS. The encapsulation efficiency was determined by the scintillation counting of an aliquot of the preparation in which traces of ³²P-end labeled ODN were added to the initial ODN. The entrapment efficiency was found to be >90% (n=10). The liposome encapsulated ODN were stored at 4°C and used within 2 weeks of preparation. Blank liposomes were prepared exactly as described above but without ODN.

Animals

Male Balb/c nu/nu mice, 10-12 weeks old, were maintained in the RRF facility of the Georgetown University according to accredited procedure and fed purina chow and water *ad libitum*.

Pharmacological disposition studies

The pharmacological disposition of free (ATG-AS) or liposome-encapsulated antisense *raf* oligodeoxyribonucleotide (LE-ATG-AS) was carried out in Balb/c nu/nu mice. Male Balb/c nu/nu mice were injected intravenously via tail vein with 30 mg/kg of ATG-AS *raf* ODN or LE-ATG-AS *raf* ODN. At 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h after injection, one animal in each group was bled from the retro-orbital sinus into heparinized

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5 tubes and sacrificed by cervical dislocation. The blood was centrifuged immediately at 2000 r.p.m for 10 min at 4°C to separate the plasma. The liver, spleen, kidney, heart and lung were rapidly excised and rinsed in ice-cold normal saline. The organs and plasma were stored frozen at -70°C until analysis.

10 ODN was isolated from plasma samples using the phenol/chloroform extraction method, and from tissues using a DNA extraction kit (Stratagene, La Jolla, CA, USA). The extracts were then loaded onto 20% polyacrylamide/8 M urea gels and electrophoresed in TBE buffer. The gel was electroblotted onto nylon membrane in 0.5X TBE buffer at 20 V for 1 h. The blots were probed with ³²P-labeled sense *raf* ODN (ATG-S *raf* ODN) in Quickhyb buffer (Stratagene, La Jolla, CA, USA) at 30°C overnight. The ODN concentration standard was prepared by spiking known amount of the ATG-AS *raf* ODN in blank plasma or blank tissue samples, followed by extraction as described above. The autoradiographs were scanned using a computer program (ImageQuant software version 3.3, Molecular Dynamics), and the amounts of ATG-AS *raf* ODN in various samples were calculated by comparison to standards.

20 SQ-20B tumor xenograft studies

25 Logarithmically growing SQ-20B cells (2×10^6) were injected subcutaneously in the flank region on both sides in male Balb/c nu/nu mice under mild anesthesia. Tumors were allowed to grow to a mean tumor volume of 115 mm³ before initiation of ODN treatment.

For intratumoral delivery of LE-ATG-AS raf ODN or LE-ATG-S raf ODN, mice were randomly divided into 3 groups. Three mice in each group received intratumoral injections of 4 mg/kg LE-ATG-AS raf ODN on the right flank, and LE-ATG-S raf ODN on the left flank. The ODN was administered intratumorally daily for 7 days. Control groups received normal saline or blank liposomes. Mice were sacrificed 24 hours after the last dose of ODN, and the organs were rapidly excised, rinsed in ice-cold normal saline and stored at -70°C until analysis. Raf-1 protein expression was analyzed from tissue homogenates by immunoprecipitation and immunoblotting and quantified using the ImageQuant computer program as described above.

Results

Effects of raf ODNs on cell viability and cell cycle distribution, and intracellular uptake

ODNs sequences were directed towards the translation initiation site of human c-raf-1 cDNA (15-mer, ends-phosphorothioated, S/As; 25-mer, fully-phosphorothioated, S-F/As-F). Since cell survival in response to γ -radiation is regulated during the cell cycle, and since several reports have suggested the possibility of a cell cycle control of Raf-1 protein kinase activity, it was important to first determine the effects of As-ODNs on the cell cycle distribution pattern of SQ-20B cells. Logarithmically growing cells were exposed to 25-100 pmol/ μ l of various ODNs for 4-12 hr, and the cell viability and cell cycle

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distribution patterns were measured. Sense and antisense ODNs showed toxicity as compared to the control cells in 1% FBS-medium without oligos. The cell cycle distributions of ODNs-treated cells were also found to differ as compared to control cells. These data are in general agreement with previous reports suggesting non-specific effects due to the introduction of oligos per se. Remarkably, ends-modified As-ODNs was neither cytotoxic nor had any significant effect on the cell cycle distribution profile of SQ-20B cells as compared to sense ODNs (S-ODNs). Similar observations were made with As-F and S-F ODNs. The intracellular uptakes of ODNs were examined. A linear increase in the intracellular level of ODNs was seen at between 2-12 hr post-treatment. Approximately 4% (equivalent to 4 pmol/ μ l) of the total extracellular or applied ODNs was taken up by these tumor cells by 12 hr. Intracellular ODNs were also found to be stable as 15-mers.

Specificity of inhibition of Raf-1.

To ascertain the specificity of inhibition of Raf-1 protein kinase by raf As-ODNs, all experiments were performed, in parallel, using two controls: i) cells were exposed to raf S-ODNs using treatment conditions identical to As-ODNs to rule out the influence of non-specific effects due to ODNs; and ii) control cells were treated with 1% FBS containing medium (without ODNs) to determine the base-line levels of the Raf-1 expression and enzymatic activity in SQ-20B cells.

The dose-dependence and time-course of inhibition of Raf-1

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protein expression by As-ODNs was studied using a combination of Raf-1 immunoprecipitation and immunoblotting assays. Densitometric analysis of the Raf-1 band (~75 kDa) showed that about 40% inhibition of Raf-1 protein expression occurred at 12 hr when 100 pmol/ μ l As-ODNs was applied to the cells. Raf-1 protein expression in the S-ODNs-treated cells (extracellular 100 pmol/ μ l, 12 hr) was found to be identical to the expression in untreated control cells, and in cells exposed to 25 pmol/ μ l extracellular As-ODNs. Specificity of the Raf-1 antibody was confirmed by elimination of the ~75 kDa band observed in control cells when immunoprecipitation was performed with antigen-blocked antibody. Time-course experiments revealed that approximately 50% inhibition of Raf-1 protein was achieved by 12 hr post-incubation with 100 pmol/ μ l extracellular As-ODNs. The inhibitory effect of As-ODNs appeared to diminish by 18 hr. This recovery of the Raf-1 expression may be attributed to the apparent degradation of As-ODNs over time and to the synthesis of new protein. Nevertheless, three independent studies indicated that approximately 50% inhibition of Raf-1 protein occurred by 12 hr in the As-ODNs-treated cells as compared to cells treated with equimolar concentration of S-ODNs.

Having established 100 pmol/ μ l as a non-toxic and inhibitory extracellular dose of *raf* As-ODNs in SQ-20B cells relative to the equimolar concentration of S-ODNs, the effect of As-ODNs on *in vitro* phosphotransferase activity of Raf-1 protein kinase was examined. Consistent with immunoblotting data, approximately 50%

inhibition of the *in vitro* Raf-1 protein kinase activity was noted in Raf-1 immunoprecipitates of As-ODNs-treated cells as compared to the S-ODNs-treated cells (applied ODNs: 100 pmol/ μ l, 12 hours) and the untreated control cells. Experiments were also performed to measure the Raf-1 immune-complex-associated *in vitro* kinase activity in tumor cells exposed to the fully modified ODNs (S-F/As-F, applied dose 100 pmol/ μ l, 12 hr). Inhibition of Raf-1 activity was observed in the As-F ODNs-treated cells as compared to the S-F ODNs response. It is noteworthy that the expression and activity of Raf-1 were observed to be similar in the control (C, without oligo) and *raf* S-ODNs-treated cells (S). This finding along with the concurrent inhibition of the expression and activity of Raf-1 noted in the *raf* As-ODNs-treated cells (As), implied that the inhibition of Raf-1 protein kinase was sequence-specific, and not due to the non-specific effects of ODNs.

***raf* As-ODNs is a biologic radiosensitizer of SQ-20B cells**

Radiation survival dose responses of SQ-20B cells exposed to S- and As-ODNs (100 pmol/ μ l, 12 hr) were evaluated. (S- and As-ODNs used in this study do not contain the G-quartet or CpG motifs previously shown to be responsible for non-antisense-specific effects such as enhanced affinity for protein or interference with the immune response.) The plating efficiencies indicated that the As-ODNs treatment had no effect on cell viability as compared to S-ODNs-treated cells (Table 1). These data are also

in agreement with the S- and As-ODNs effects on the viability of logarithmically growing cells discussed earlier. Radiation survival dose responses of the control (without oligo) and S-ODNs-treated cells were almost identical. Most important, As-ODNs treatment resulted in decreases of the shoulder and the slope of the survival curve. The radiobiological parameters were obtained by fitting the data (surviving number of colonies) to the single-hit multitarget (D_0 , D_q , n) and linear-quadratic (α , β) models of radiation survival response. In addition, the value of a model-free parameter, mean inactivation dose (D) was calculated (14) (Table 1). Based on a ratio of the mean inactivation dose, the dose modifying factor (DMF) of As-ODNs treatment was 1.4. Significant decreases observed in the values of radiobiological parameters, D , D_q , and D_0 of SQ-20B cells following treatment with the *raf* As-ODNs indicate a good correlation between the DNA sequence-specific inhibition of Raf-1 protein kinase and the radiosensitization of these relatively radioresistant tumor cells.

The studies indicated that greater than 50% inhibition of Raf-1 expression could be achieved with only 10 $\mu\text{mol} \cdot \mu\text{l}$ of the liposome-encapsulated *raf* As-ODNs.

Liposomal encapsulation protects ATG-AS *raf* ODN *in vivo*

The plasma concentration-time profile of LE-ATG-AS *raf* ODN was studied. Dosage of 30 mg/kg LE-ATG-AS *raf* ODN or ATG-AS *raf* ODN was administered i.v. in Balb/c nu/nu mice. Blood samples were collected from retro-orbital sinus at indicated times after

injection and the ODN in plasma samples was extracted by phenol:chloroform. The samples were electrophoresed on 20% polyacrylamide/8 M urea gel and electroblotted on nylon membrane. The blots were probed with ^{32}P -labeled ATG-S. *raf* ODN. Auto-radiographs were scanned using a computer program (ImageQuant software version 3.3, Molecular Dynamics). St, standard prepared by spiking known concentration of ATG-AS *raf* ODN in blank plasma. Quantification data were calculated based on a known concentration of the standard sample, and then normalized against dilution factors at various time points.

Following intravenous administration, the peak plasma concentration of 6.39 $\mu\text{g}/\text{ml}$ was achieved and intact ODN could be detected up to 24 h. The decrease in plasma concentration of LE-ATG-AS *raf* ODN followed a biexponential pattern with an initial half-life ($t_{1/2\alpha}$) of 24.5 min and a terminal half-life ($t_{1/2\beta}$) of 11.36 h. The area under the plasma concentration-time curve for LE-ATG-AS *raf* ODN was 5.99 $\mu\text{g}\cdot\text{h}/\text{ml}$, with total body clearance of 75.94 $\text{ml}/\text{min}/\text{kg}$ and volume of distribution of 74.67 L/kg . In contrast, intact free ODN (ATG-AS *raf* ODN) was detectable only at 5 min; the plasma concentration being 9.75 $\mu\text{g}/\text{ml}$. These observations indicate that ATG-AS ODN was either rapidly cleared from the circulation, or extensively degraded in plasma due to nuclease activity.

Normal tissue distribution profiles of LE-ATG-AS *raf* ODN were studied. Tissue samples were collected at indicated times

after i.v. administration of 30 mg/kg LE-ATG-AS *raf* ODN. ODN was extracted from homogenized tissues using a DNA extraction kit (Stratagene). Samples were electrophoresed and electroblotted. The blots were probed with ³²P-labeled ATG-S *raf* ODN and autoradiographs were analyzed. Quantification data were calculated based on a known concentration of the standard sample, and then normalized against the weight of the tissue sample collected.

Intact ODN could be detected in all organs examined up to 48 hours after administration. Interestingly, following ATG-AS *raf* ODN administration, intact ODN could be detected only at 5 minutes post-administration in all the organs examined and degradation products were observed at all other times. These findings, along with the plasma data for LE-ATG-AS *raf* ODN, suggests that ODN with only the end bases phosphorothioated is rapidly degraded *in vivo* and that liposome encapsulation using the liposomes of the invention protects it from degradation for at least 48 hours.

Table 1 Radiation survival parameters of SQ-20B cells treated with raf oligodeoxyribonucleotides

raf ODNs	No. of Expts.	D ₀ (Gy)	D _q (Gy)	n	α (Gy ⁻¹)	β (Gy ⁻²)	D
Sense/Control*	6	3.028	1.265	1.519	0.2373	0.0050	3.694
Antisense	3	2.374	0.551	1.261	0.3614	0.0038	2.628

The appropriate number of cells were seeded into two replica T-25 flasks per dose in each experiment. Control cells were cultured in medium containing 1% FBS without oligo. Plating efficiencies of the S-ODNs-treated, As-ODNs-treated and control cells were in the range of 13-58%, 28-61% and 41-67%, respectively. Clonogenic survival data were computer-fitted to the single-hit multitarget and the linear-quadratic models of radiation survival dose response. Composite values of the various parameters were obtained from the three experiments performed with the S-ODNs-treated and the three experiments using the control cells.

Specificity of inhibition of Raf-1 protein expression and activity in vitro

Initially, the possibility of cytotoxic effects of liposomes in SQ-20B cells was examined. Blank liposomes, at concentration equivalent to 10 μ M LE-ATG-AS raf ODN, were found to be non-cytotoxic as determined by the clonogenic and trypan blue dye exclusion methods. However, blank liposomes showed cytotoxicity at doses higher than 20 μ M. Therefore, a dose of 10 μ M or less was used for in vitro experiments. Further, 10 μ M LE-ATG-AS raf ODN or LE-ATG-S raf ODN was non-toxic to SQ-20B cells.

Specificity of inhibition of Raf-1 protein expression by LE-ATG-AS raf ODN. Logarithmically growing SQ-20B cells were treated with 10 μ M LE-ATG-AS raf ODN (AS), 10 μ M LE-ATG-S raf ODN (S) or blank liposomes (BL) for indicated time in 1% FBS containing medium. Untreated control cells (C) were simultaneously switched to 1% FBS containing medium for 8 hours. Whole cell lysates were normalized for total protein content and immunoprecipitated with agarose-conjugated polyclonal anti-Raf-1 antibody (Santa Cruz). Immune-complexes were resolved on 7.5% SDS-PAGE and Raf-1 protein expression was detected by immunoblotting with polyclonal anti-Raf-1 antibody (Santa Cruz), followed by the ECL detection protocol (Amersham). Results from three independent experiments were quantified using a computer program (ImageQuant, Molecular Dynamics), and data are expressed relative to the level of Raf-1 in LE-ATG-S raf ODN-treated cells

(bottom). Panel B: Dose-response analysis. Logarithmically growing SQ-20B tumor cells were treated with indicated concentrations of LE-ATG-AS *raf* ODN (AS) or LE-ATG-S *raf* ODN (S) in 1% FBS containing medium for 8 h. Normalized cell lysates were analyzed for Raf-1 protein expression.

Logarithmically growing SQ-20B cells were treated with 10 μ M LE-ATG-AS *raf* ODN (AS), or 10 μ M LE-ATG-S *raf* ODN (S) for 8 h in 1% FBS containing medium. Control cells (C) were simultaneously switched to 1% FBS containing medium for 8 hours. Whole cell lysates were normalized for protein content, and Raf-1 was immunoprecipitated. Phosphotransferase activity of Raf-1 in immune-complexes was assayed *in vitro* using a physiologic substrate MKK1. Radiolabeled reaction products were separated by electrophoresis and autoradiographed.

Time-course experiments revealed that a maximum inhibition ($52.3 \pm 5.7\%$) of Raf-1 protein expression (~ 74 kDa) occurred at 8 hours post-incubation of cells with 10 μ M LE-ATG-AS *raf* ODN. The inhibitory effect of LE-ATG-AS *raf* ODN was maintained up to 24 h ($45.6 \pm 9.8\%$). The level of Raf-1 protein was comparable in the control untreated cells (C), blank liposome-treated cells (BL), and LE-ATG-S ODN-treated cells, indicating the LE-ATG-AS *raf* ODN specifically inhibited the Raf-1 protein expression in SQ-20B cells. Dose response studies showed that $35.94 \pm 16.8\%$ and $52.3 \pm 5.7\%$ inhibition of Raf-1 expression occurred with 5 μ M and 10 μ M LE-ATG-AS *raf* ODN treatment for 8 hours.

5 The effects of LE-ATG-AS *raf* ODN on the enzymatic activity of Raf-1 protein kinase using mitogen-activated protein kinase kinase (MKK1) as substrate were studied. In concurrence with Raf-1 protein inhibition data, it was found that 10 μ M LE-ATG-AS *raf* ODN treatment for 8 hours inhibited $62.6 \pm 9.0\%$ *in vitro* phosphotransferase activity of Raf-1 protein. LE-ATG-S *raf* ODN did not have any effect on the Raf-1 protein kinase activity as compared with the untreated control cells.

LE-ATG-AS *raf* ODN is a biological radiosensitizer

10 Radiation survival dose responses of SQ-20B cells exposed to LE-ATG-AS *raf* ODN, LE-ATG-S *raf* ODN, and blank liposomes are presented in Table 2. Comparison of the radiation survival dose response of SQ-20B cells treated with LE-ATG-AS *raf* ODN (AS), LE-ATG-S *raf* ODN (S), or blank liposomes (BL). The appropriate number of cells were seeded in duplicate to obtain 40-60 colonies per T-25 flask (Costar) for each radiation dose. The clonogenic survival data were computer-fitted to the single-hit multitarget model of radiation survival dose response. Representative data from one experiment performed for each treatment category were evaluated.

20 The plating efficiencies of cells treated with S/AS ODN or blank liposomes were comparable (Table 2). Radiation survival dose responses of the blank liposome-treated (BL) and LE-ATG-S *raf* ODN-treated cells were also comparable. LE-ATG-AS *raf* ODN treatment resulted in a significant radiosensitization (Table 2).

Based on a ratio of the mean inactivation dose, the dose modifying factor (DMF) of LE-ATG-AS *raf* ODN treatment was 1.6. Significant decreases observed in the values of radiobiological parameters, \bar{D} , D_q , and D_0 of SQ-20B cells following treatment with the LE-ATG-AS *raf* ODN indicate a good correlation between the DNA sequence-specific inhibition of Raf-1 protein kinase and the radiosensitization of these relatively radioresistant tumor cells.

LE-ATG-AS *raf* ODN is a specific inhibitor of Raf-1 protein expression in solid tumor

The effects of intratumoral administration of LE-ATG-AS *raf* ODN and LE-ATG-S *raf* ODN on the expression of Raf-1 protein was examined in a SQ-20B tumor xenograft model. Mice with tumors on both flanks received intratumorally LE-ATG-AS *raf* ODN on the right flank, and LE-ATG-S ODN on the left flank. Results demonstrated a significant inhibition of Raf-1 protein in tumor tissue following treatment with LE-ATG-AS *raf* ODN compared with LE-ATG-S *raf* ODN ($60.3 \pm 6.4\%$).

Inhibition of Raf-1 protein expression by LE-ATG-AS *raf* ODN in SQ-20B tumor xenografts

SQ-20B tumors were established subcutaneously in both hind limbs of nude mice, Balb C nu/nu (mean tumor volume 115 mm^3). Each animal then received intratumoral injections of LE-ATG-AS *raf* ODN (AS) on the right flank and LE-ATG-S *raf* ODN (S) on the left flank at a dose of 4 mg/kg daily for 7 days. Tumor tissue

was excised 24 h after the last treatment, and Raf-1 protein expression in tumor samples was analyzed. ECL images were quantified using a computer program (ImageQuant, Molecular Dynamics), and quantification data from three mice are expressed as the level of Raf-1 protein expression in LE-ATG-AS raf ODN-treated tumors relative to LE-ATG-S raf ODN-treated tumors.

Liposomes prepared in accord with the teachings of the invention are non-toxic both in culture and in animals. While only specific liposomes are disclosed herein, the novel carriers may be used for delivery of a variety of DNA-based compounds for delivery.

Table 2. Radiation Survival Parameters of SQ-20B Cells Treated with LE-ATG-S/AS raf ODN

raf ODN	No. of Expts.	D ₀ (Gy)	D _q (Gy)	\bar{n}	α (Gy ⁻¹)	β (Gy ⁻²)	\bar{D} (Gy)
Blank liposomes/	5	2.795	1.445	2.012	0.2184	0.0087	3.659
LE-ATG-S*		± 0.38	± 1.22	± 1.34	± 0.11	± 0.00	± 0.02
LE-ATG-AS	3	2.287	0.051	1.021	0.4385	0.0000	2.280
		± 0.23	± 0.05	± 0.19	± 0.05	± 0.00	± 0.00

The appropriate number of cells were seeded in duplicate T25 flasks per dose in each experiment. Plating efficiencies of the blank liposome-treated, LE-ATG-S raf ODN-treated, and LE-ATG-AS raf ODN-treated cells were in the range of 65-79%, 52-83%, and 59-90% respectively. Clonogenic survival data were computer-fitted to the single-hit multitarget and the linear-quadratic models of radiation survival dose response.

*Composite values of the various parameters were obtained from the three experiments performed with LE-ATG-S raf ODN-treated cells and two experiments performed with the blank liposome-treated cells.

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5 The liposomes of the invention provide significant protection of antisense oligonucleotides against degradation in blood and normal tissue. The formulation may replace the need for complete modification of all bases of the antisense oligonucleotides for therapeutic uses. Compositions comprising oligonucleotides may be administered in many ways, depending on the target tissue.

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tered systemically. A preferred method of administration is by intravenous injection.

Acceptable carriers include, for example, glucose, saline, phosphate buffered saline. Carriers may also contain other substances frequently found in pharmaceuticals such as preservatives, emulsifiers and surfactants.

Dosage will depend on the extent to which it is possible to present the active agents to the target tissue. The appropriate dosage should deliver a serum concentration of about 1 $\mu\text{g/ml}$ to 1000 $\mu\text{g/ml}$. In some instances, this dosage can be delivered into the target tissue directly. Hence, that level of dosage need not be achieved in the total blood volume.

What we claim is:

1. A composition comprising a cationic liposome containing a cationic lipid, phosphatidylcholine and cholesterol.
2. A composition of claim 1 wherein the liposome contains an antisense oligonucleotide sequence.
3. A composition of claim 2 wherein the antisense sequence is a raf oligodeoxynucleotide.
4. A composition of claim 3 wherein the antisense sequence is of the formula 5'-GTGCTCCCATGATGC-3' wherein only the terminal sequences are phosphorothioated.
5. A composition of claim 1 in a pharmaceutically acceptable carrier.
6. A composition of claim 4 in a pharmaceutically acceptable carrier.
7. A composition of claim 1 wherein the pharmaceutically acceptable carrier is isotonic.
8. A composition of claim 4 wherein the pharmaceutically

acceptable carrier is a buffered, isotonic solution.

9. A method of radiosensitizing tumor tissue by administration of a radiosensitizing effective amount of at least one antisense oligonucleotide of no more than 40 bases containing the sequence 5'-GTGCTCCATTGATGC-3'.
10. A method of claim 9 wherein the oligonucleotide is phosphorothioated at only the end nucleotides.
11. A method of claim 9 wherein the oligonucleotide is phosphrothioated at only the end nucleotides.
12. A method of claim 9 wherein the oligonucleotide is administered intravenously.
13. A method of claim 9 wherein the oligonucleotide is administered directly to the target tissue.
14. A method of claim 9 wherein the oligonucleotide is administered into the arterial supply to the target tissue.
15. A method of claim 9 wherein the oligonucleotide is of the formula 5'-GTGCTCCATTGATGC-3' and only the end bases only are phosphorothioted.

16. A composition of matter comprising liposomes containing the sequence 5'-GTGCTCCATTGATGC-3' in a pharmaceutically acceptable carrier.
17. A composition of claim 1 wherein the cationic lipid is dimethyldioctadecyl ammonium bromide.

ABSTRACT

It is possible to radiosensitize tumor cells by administration of compositions containing the Human antisense c-raf-1 oligodeoxyribonucleotide (ODN/oligo) sequence:

5'-GTGCTCCATTGATGC-3' (seq. #1) wherein only the end bases are phosphorylated is a preferred embodiment. Antisense sequences of up to 40 bases which containing this sequence may be used in accord with the teachings of this disclosure. Compositions comprising a cationic liposome of dimethyldioctadecyl ammonium bromide, phosphatidylcholine and cholesterol may be used as a carrier system. The liposomes provide a new carrier system that is particularly useful for administration of sequences for therapy.

0157327-102497

DECLARATION AND POWER OF ATTORNEY

As below named inventors, We hereby declare that:

We believe we are the original, first and sole inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled : "LIPOSOMES CONTAINING OLIGONUCLEOTIDES", which is filed herewith.

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37 CFR 1.56(a).

We hereby claim the benefit of priority on basis of the Provisional Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. 112, we acknowledge the duty to disclose material information as defined in 37 CFR 1.56(a) which occurred between the filing date of the prior application and the filing date of this application:

Prov. Patent Appl. 60/041,192 Filing date: March 21, 1997

We hereby appoint the following attorneys and agents, with full power of substitution, association, and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Glenna Hendricks, Reg. No. 32,535
Carol Tracy, Reg. No. 34,976

Address all telephone calls to Glenna Hendricks. Please address all correspondence to:

Glenna Hendricks
Hendricks and Associates
P.O. Box 2509
Fairfax, VA 22031-2509 Telephone (703) 591-4470

We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Usha Kasid
Inventor

Usha Kasid
Inventor's signature

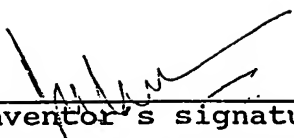
10/17/97
date

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Citizenship

7/15/99

Prafulla Gokhale
Inventor


Inventor's signature


10/13/97
date

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Home address
India

Citizenship

Anatoly Dritschilo
Inventor


Inventor's signature

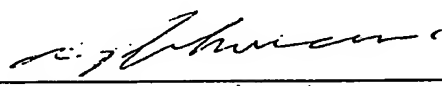
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date

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Citizenship

Aquilur Rahman
Inventor


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10.14.97
date

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U.S.A.

Citizenship

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Application of
Kkasid, et al.
USPN 08/957,327
Filed: October 24, 1997
Title: LIPOSOMES CONTAINING OLIGONUCLEOTIDES

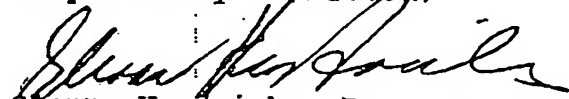
ASSOCIATE POWER OF ATTORNEY

Honorable Commissioner of Patents June 4, 1999
and Trademarks
Washington, D.C. 20231

Sir:

As an attorney of record in the cited application, I hereby grant to Robin Teskin, Reg. No. 35,030, an associate power of attorney to transact business associated therewith before the Patent and Trademark Office.

Respectfully Submitted;


Gienna Hendricks, Reg. No. 32535

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Kasid, et al.
Patent Application filed herewith
Filed:
For: LIPOSOMES CONTAINING OLIGONUCLEOTIDES

DECLARATION CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9 (f) & 1.27(d))--NONPROFIT ORGANIZATION

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I hereby declare that I am an official empowered to act on behalf
of the nonprofit organization identified below:

Georgetown University

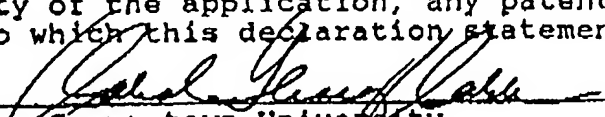
Washington, D.C.

The above named institution is an institution of higher learning.
I hereby declare that the nonprofit organization identified above
qualifies as a nonprofit organization as defined in 37 CFR 1.9(e)
for purposes of paying reduced fees to the United States Patent
and Trademark Office regarding the invention entitled :

LIPOSOMES CONTAINING OLIGONUCLEOTIDES

I acknowledge the duty to file, in this application or patent,
notification of any change in status resulting in loss of enti-
tlement to small entity status prior to paying, or at the time of
paying, the earliest issue fee or any maintenance fee due after
the date on which status as a small entity is no longer ap-
propriate.

I hereby declare that all statements made herein of my own
knowledge are true and that all statements made on information
and belief are believed to be true; and further that those state-
ments were made with the knowledge that willful false statements
and the like so made are punishable by fine or imprisonment, or
both, under section 1001 of Title 18 of the United States Code;
and that such willful false statements may jeopardize the valid-
ity of the application, any patent issuing thereon, or any patent
to which this declaration statement is directed.


For Georgetown University
37th and O Streets, N.W., Washington, D.C. 20057

10/21/97
Date

Address

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	
)	
Usha KASID et al.)	Group Art Unit: Unassigned
)	
Application No. Unassigned)	Examiner: Unassigned
)	
Filed: July 15, 1999)	
)	
For: LIPOSOMES CONTAINING)	
OLIGONUCLEOTIDES)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified patent application, kindly amend the disclosure as follows:

IN THE CLAIMS

Kindly cancel Claims 1 to 17 (all original claims) and substitute the following claims therefor:

--18. An improved method of treatment which includes the administration of therapeutic radiation wherein the improvement comprises the administration of at least one oligonucleotide in an amount effective to enhance the efficacy of said therapeutic radiation.

11/15/99

19. The method of Claim 18 wherein said method is used for the treatment of cancer.
20. The method of Claim 18 wherein said method is used for the treatment of a tumor that is resistant to radiation therapy.
21. The method of Claim 18 wherein said at least one oligonucleotide is encapsulated in a liposome.
22. The method of Claim 18 wherein said at least one oligonucleotide is up to about 40 nucleotides in length.
23. The method of Claim 18 wherein said oligonucleotide is modified.
24. The method of Claim 18 wherein said at least one oligonucleotide comprises to a portion of an oncogene or greater factor gene.
25. The method of Claim 24 wherein said oncogene is selected from the group consisting of *ras*, *raf*, *cot*, *mos*, and *myc* genes.

26. The method of Claim 24 wherein said growth factor gene is a PDGF or FGF gene.

27. The method of Claim 21 wherein said liposome is a cationic liposome.

28. The method of Claim 27 wherein said cationic liposome is comprised of dimethyldioctadecyl ammonium bromide, phosphatidylcholine and cholesterol.

29. The method of Claim 18 wherein said amount of said at least one oligonucleotide is administered intratumorally.

30. The method of Claim 18 wherein said at least one oligonucleotide is administered in the form of a composition comprising an amount thereof of about 10pmol/ μ l or greater.

31. The method of Claim 18 wherein said at least an oligonucleotide is an antisense DNA encoding a portion of the *raf* oncogene.

32. The method of Claim 31 wherein said antisense DNA has SEQ. ID. NO. 1 or SEQ. ID. NO. 2.

33. The method of Claim 18 wherein said oligonucleotide is administered by injection.--

REMARKS

Favorable consideration on the merits is respectfully requested. Applicants respectfully advise that the claims are all directed to methods for improving the efficacy of therapeutic radiation-based therapies.

These methods have particular application in the treatment of radiation-resistant tumor. Quite surprisingly, it has been discovered that the inclusion of the administration of an oligonucleotide during therapeutic radiation synergistically enhances the efficacy thereof. This is surprising and the actual mechanism by which this occurs is unclear, however is believed to involve the induction of apoptosis.

Therefor, the present invention provides a significant advance in the cancer therapy as it will provide for radiation to be effective, or more effective, in treatments wherein it has little or no efficacy, e.g., radiation resistant tumors.

Application No. Unassigned
Attorney's Docket No. 010091-056

If the Examiner has any questions in connection with this application he or she is respectfully requested to contact the undersigned so that prosecution may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: Robin L. Teskin
Robin L. Teskin
Registration No. 35,030

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

Date: July 15, 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	
)	
Usha KASID et al.)	Group Art Unit: Unassigned
)	
Application No.: Unassigned)	Examiner: Unassigned
)	
Filed: July 15, 1999)	
)	
For: LIPOSOMES CONTAINING)	
OLIGONUCLEOTIDES)	

STATEMENT PURSUANT TO 37 C.F.R. §1.822

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants hereby advise that this application contains the identical sequence disclosure as Serial No. 08/957,327, filed October 24, 1997 (the parent of this application). Accordingly, as paper and computer readable forms of the Sequence Listing have been filed in said parent application, a duplicate need not be provided herein, pursuant to the provisions of 37 C.F.R. § 1.1822(e). Applicants respectfully request that a computer readable form be generated corresponding to the sequence listing provided in the parent application.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Kasid, Usha
Gokhale, Prafulla
Dritschilo, Anatoly
Rahman, Aqulur
- (ii) TITLE OF INVENTION: Liposomes containing Oligonucleotides
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Hendricks and Assoc.
 - (B) STREET: P.O. Box 2509
 - (C) CITY: Fairfax
 - (D) STATE: VA
 - (E) COUNTRY: US
 - (F) ZIP: 22031
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/957,327
 - (B) FILING DATE: 24-OCT-1997
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Hendricks, Glenna
 - (B) REGISTRATION NUMBER: 32,535
 - (C) REFERENCE/DOCKET NUMBER: kasid
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (703) 591-4470
 - (B) TELEFAX: (703) 591-4428

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTGCTCCATT GATGC

15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTGTATGTG CTCCATTGAT GCAGC

25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

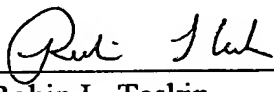
GCAATCAATGG AGCAC

15

For the convenience of the Office, a copy of the Sequence Listing is provided
herewith.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: 
Robin L. Teskin
Registration No. 35,030

Post Office Box 1404
Alexandria, VA 22313-1404
(703) 836-6620

Date: July 15, 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	
)	
Usha Kasid et al.)	
)	
Application No.: Unassigned)	Group Art Unit: Unassigned
)	
Filed: July 15, 1999)	Examiner: Unassigned
)	
For: LIPOSOMES CONTAINING)	
OLIGONUCLEOTIDE)	

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with the duty of disclosure as set forth in 37 C.F.R. §1.56,
Applicants hereby submit the following information in conformance with 37 C.F.R.
§§ 1.97 and 1.98. Pursuant to 37 C.F.R. § 1.98, a copy of the document cited is
enclosed.

The document is being submitted within 3 months of the filing or entry of the
national stage of this application or before the first Office Action on the merits,
whichever is later, therefore no fee or certification is required under 37 C.F.R.
§ 1.97(b).

11/15/99

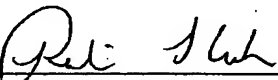
INFORMATION DISCLOSURE CITATION PTO-1449				ATTORNEY'S DKT NO. 010091-056		APPLICATION NO. Unassigned	
				APPLICANT Usha Kasid et al			
				FILING DATE July 15, 1999		GROUP Unassigned	
U.S. PATENT DOCUMENTS							
EXAMINER'S INITIALS	PATENT NO.	DATE	NAME	CLASS	SUBCLASS	FILING DATE	
	5,756,122	05/26/98	Thierry et al.				
FOREIGN PATENT DOCUMENTS							
EXAMINER'S INITIALS	PATENT NO.	DATE	COUNTRY	CLASS	SUBCLASS	Translation	
						Yes	No
OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)							
EXAMINER				DATE CONSIDERED			

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

To assist the Examiner, the document is listed on the attached form PTO-1449.
It is respectfully requested that an Examiner initialled copy of this form be returned to
the undersigned.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: 
Robin L. Teskin
Registration No. 35,030

P.O. Box 1404
Alexandria, Virginia 22313-1404
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Date: July 15, 1999